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S U B ~~P R O P O S A L~~ REPORT B I O L O G I C A L C H E M I S T R Y

A 0500/3057

21-DAY INHALATION STUDY WITH

MAIN AND SIDESTREAM CIGARETTE SMOKE OF

STANDARD REFERENCE CIGARETTE 2R1

ON RATS

BIOCHEMICAL AND MORPHOLOGICAL FREE LUNG CELL RESPONSES

(PT)

2029027195

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ABBREVIATIONS (a,b)

ABT : American Board of Toxicology

ACP	: acid phosphatase
approx.	: approximately
Apr.	: April
Aug.	: August
BC	: Biological Chemistry
Bis	: N,N'-methylene-bis-acrylamide
BSA	: bovine serum albumin
BW	: body weight
Dec.	: December
DNA	: deoxyribonucleic acid
EC	: enzyme code according to the "International Union of Biochemistry Commission on Enzymes"
EDTA	: ethylenediaminetetraacetic acid
equiv.	: equivalent
Feb.	: February
Fr.	: Friday
Frel	: relative fluorescence units
.GT:	: greater than
x g	: centrifugal force in terms of the constant of gravitation (1 x g = 9.81 m/s2)
Hepes	: N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid
i.p.	: intraperitoneal
Jan.	: January
Jul.	: July
Jun.	: June
Mar.	: March
max.	: maximal
MEM	: minimal essential medium
Mo.	: Monday
4-MUP	: 4-methylumbelliferyl phosphate
~~no.~~, ~~No.~~	: number

(a) in addition to those, which are explained immediately on the same page

(b) Units are given in accordance with SI units (Système International d'Unités).

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ABBREVIATIONS (continued)
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Nov. : November
Oct. : October
PBS : phosphate-buffered saline
~~pH : negative-decadic-logarithm of hydrogen-ion concentration~~
~~rpm : revolutions per minute~~
~~RT : room temperature~~
Sa. : Saturday
SDS : sodium dodecyl sulfate
Sep. : September
Su. : Sunday
TEMED: N,N,N',N'tetramethylethylendiamine
Th. : Thursday
Tris : tris(hydroxymethyl)aminomethane
Tu. : Tuesday
~~U : unit(s)~~
~~vs : versus~~
We. : Wednesday

Ex: X as exponent to the Base 10, e.g. E2 = 10^2

SUMMARY

1 SUMMARY

In the present 21-day inhalation study the influence of SIDESTREAM and MAINSTREAM CIGARETTE SMOKE of the standard reference cigarette type 2R1 on selected responses of free lung cells (FLC) was investigated.
The objectives of this study were:
(1) the application of flow cytometry in combination with or in place of conventional FLC methods and
(2) the selection of a reproducible lavage method for future studies.

144 rats were randomly allocated to 3 groups, 60 for sham control, 60 for mainstream smoke and 24 for sidestream smoke exposure. The head-only exposure was performed for 420 minutes/day at 0.28 milligrams/liter total particulate matter (TPM) corresponding to a daily dose of 118 minutes x milligram/liter TPM for mainstream smoke and at 0.07 milligrams/liter TPM corresponding to a daily dose of 29 minutes x milligram/liter TPM for sidestream smoke. Rats were exposed 7 days/week for a period of 21 days. This study was divided into 3 subsequent experiments. Sidestream exposure only took place in the 3rd experiment.

FLC were harvested after a nonrecycling lavage using 10 to 13 cycles. As the composition of FLC populations is strongly influenced by the number of lavage cycles and the constituents of lavage medium, both of these parameters were varied in this study. The lavage media used were:
(1) phosphate-buffered saline (PBS),
(2) PBS plus bovine serum albumine (BSA) and
(3) PBS plus calcium and magnesium.

FLC were investigated by light microscopic and flow cytometric methods using intact cells. In addition biochemical parameters were assayed in FLC homogenates.

Biochemical parameters assayed in FLC homogenates were:
(1) protein pattern after separation by electrophoresis,
(2) phospholipid content/macrophage and
(3) specific activity of acid phosphatase.

The FLC parameters assayed by light microscopy were:
(1) proportion of macrophages, granulocytes and lymphocytes,
(2) number of macrophages/rat,
(3) viability of macrophages and
(4) macrophage nucleus and vacuole size.

The FLC parameters assayed by flow cytometry were:
(1) phagocytotic activity of pulmonary macrophages,
(2) DNA content,
(3) viability and nonspecific esterase activity as well as
(4) differentiation of subpopulations according to light scatter properties (size, structure).

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Forschung GmbH

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RESPONSIBILITY

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2 RESPONSIBILITY

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INTRODUCTION

3 INTRODUCTION

The reponses of FLC to whole cigarette smoke inhalation in previous studies (a) demonstrated that these easily accessible lung cells can be a sensitive indicator for the composition and the dose of inhaled cigarette smoke. ~~Similar and additional~~ Macrophage responses to cigarette smoke ~~were~~ published during recent years, e. g. the effects on number (Smith et al., 1978), migration into lungs (Matulionis, 1979a and b), phagocytosis (Drath et al., 1978), chemotaxis (Warr and Martin, 1973, Demarest et al., 1979), surface morphology and receptors (Warr and Martin, 1977, Davis et al., 1980), ultrastructure (Pratt et al., 1971, Lewis et al., 1979), oxidative metabolism (Drath et al., 1978, York et al., 1973), protein synthesis (Holt and Keast, 1973, Leffingwell and Low, 1979), glyceraldehyde 3-phosphate dehydrogenase activity (Powell and Green, 1972) and lysosomal enzyme content (Martin, 1973, Warr and Martin, 1978, Scharfman et al., 1980).

It has been reported that the physical variables in the procedures of lung lavage and harvest of free lung cells, e. g. pressure, volume, retention time and/or temperature of the instilled lavage medium may not only lead to differences in the number and type of free lung cells obtained, but also in their morphology and viability as well as in their biochemical and functional characteristics (e. g. Brain and Frank, 1968, Holt, 1979). As free lung cell responses were found to vary considerably in previous studies and methodological variations were a probable source of this problem, a nonrecycling lavage procedure, which also allows a fractionation of free lung cells with successive lavage cycles, was developed (b) and tested in previous studies.

The characteristics of FLC have also been reported to depend on the composition of the lavage medium used (Holt, 1979, Kavet and Brain, 1977). A previous study (b) using PBS (c), PBS supplemented with

(a) see REFERENCES: INBIFO studies A -/3013, A -/3018 and A -/3025
(b) see REFERENCES: INBIFO study A -/3047
(c) calcium and magnesium free PBS

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newborn calf serum (a) or with bovine serum albumin (BSA) (b,c) as lavage media demonstrated an almost 100-fold difference in the number of cells lavaged per rat and significant changes in cell populations and viability. It could be concluded that the addition of serum to medium is only ~~important~~ for the removal of granulocytes ~~(PMN)~~ and other nonadherent particles in the 1st few cycles and that BSA supplementation could be useful in increasing the yield of free lung cells ~~from each~~ per rat ~~for examination.~~ It appears to be advantageous, however, to supplement PBS with calcium and magnesium for a "prelavage" instead of serum to avoid the possibility of immunological influences due to serum antigens. These data, however, are only preliminary as they were derived from only 1 or 2 experiments. The questions of reproducibility of the effects seen and the influence of supplements to the lavage medium on FLC responses, e. g. phagocytosis, however, ~~was~~ were investigated in the present study.

Some of the FLC responses were assayed by flow cytometry. This automated cell analysis and sorting technique provides a new approach for rapid and simultaneous determination of biochemical and physical properties of a large number of cells individually (e. g. 1E5 cells). The samples fixed and/or stained with fluorescent dyes specific for different biochemical parameters are analyzed in liquid suspension as they flow through a chamber intersecting a laser beam. Multiple sensors determine fluorescence and light scatter on a cell-by-cell basis. Cellular parameters which are proportional or related to these optical parameters are DNA content, total protein, cytoplasmic granulation (fine structures), nuclear and cytoplasmic diameter among others.

The flow cytometer (FCM) employed in this study is capable of

(a) 50 milliliters/liter
(b) see REFERENCES: INBIFO studies A -/3047 and A -/3056
(c) protein concentration equivalent to that with 50 milliliters/liter serum, approx. 3 grams/liter

however, do not accumulate fluorescein to any appreciable extent, although they may be capable of hydrolyzing FDA. The biokinetics of FDA conversion by cells is rather complex as it involves many steps ~~like~~ FDA influx, binding to cellular components, enzymatic hydrolysis, product inhibition, binding of fluorescein to intracellular components, an efflux of free fluorescein. Under suitable conditions cells may reach a steady state which, as a rule, is almost exclusively depending on the efflux rate of fluorescein. The efflux rate, in turn, is a function of membrane permeability. FDA has therefore been proposed as a probe for cell viability and damage manifesting itself as leaky cell membrane (Rotman and Papermaster 1966, Augsten and Güttner 1975, Back et al., 1973 and Baisch 1978). The nonspecific esterase activity with FDA must therefore not be confused with the nonspecific esterase known from cytochemistry, which usually is determined on fixed cells with other substrates. The incubation of cells with FDA is more or less a way of smuggling fluorescein into viable cells in order to obtain information on the functional state of their membrane.

The use of FWD versus right angle light scatter (RAS) of cells in suspension has been described to differentiate between various cell subpopulations with differing morphology, i. e. size and fine structure (Salzman et al., 1975). As FLC, and especially pulmonary macrophages, demonstrate large variations in cell morphology (Finch et al., 1982), this method can be applied to the various lavaged pools of sham and smoke-exposed FLC. Changes in cell size ~~will~~ were also be determined by planimetry on cytocentrifuge preparations using a digitizer.

Phagocytosis of particles by pulmonary macrophages has been shown to increase after smoke exposure in previous experiments (a). The method used to date, however, is cumbersome and dependent on cell adhesion, which is also reported to vary after smoke exposure (Mann et al., 1971 and Rasp et al., 1978). The determination of FLC

(a) see REFERENCES: INBIFO studies A -/3013, A -/3018 and A -/3025

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analyzing and storing up to 4 correlated parameters per cell. Frequency distributions for 1 (histogram) or any combination of 2 correlated parameters (cytogram) can be displayed for computer evaluation.

Methods for the determination of DNA content (Barlogie et al., 1979) and viability of single FLC have already been tested with a fluorescence-activated cell sorter (FACS 2) (a).

The trypan blue assay is probably the most widely employed microscopic viability assay and has also been used in the present study (see). This assay is based on the principle of dye exclusion by viable cells as opposed to dye uptake by nonviable cells. Counting of stained and unstained cells can also be performed by FCM using FWD and AXL (Adams, 1977). As these 2 parameters were used for the differentiation of macrophages and granulocytes in all FCM determinations, the application of FCM to the trypan blue assay was not considered to be appropriate in the present study. Instead, the fluorescent DNA stain propidium iodide (PI) was used. Like trypan blue, this stain is excluded from viable cells, but penetrates the membrane of nonviable cells thus inducing red nuclear fluorescence. The nonspecific esterase activity of FLC was determined simultaneously as it not only provides enhanced and more detailed information on viability but also may further differentiate between FLC subpopulations. The esterase activity was determined after incubation of FLC with the nonfluorescent substrate fluorescein diacetate (FDA). Due to its nonpolar character FDA is readily taken up by both viable and nonviable cells. A variety of so-called nonspecific esterases, which are ubiquitous in nucleated cells, then hydrolyze FDA yielding acetate and green fluorescent fluorescein. As a polar substance, fluorescein is retained within viable cells with intact membranes and may accumulate several 100-fold over the extracellular FDA concentration ( ). Nonviable cells,

(a) see REFERENCES: INBIFO study A -/3056

Einochs (A)

The present study was the 1st application of this instrument to determine biological effects of SS and MS smoke. Due to repeated failure of the laser system, only (3) and (4) were performed.

This automated cell analysis and sorting technique provides a new approach for rapid and simultaneous determination of biochemical and physical properties of single cells at a usual rate of 500 cells/second. Individual samples fixed and/or stained with fluorescent dyes specific for different biochemical parameters are analyzed in liquid suspension as they flow through a chamber intersecting a laser beam. Multiple sensors determine fluorescence and light scatter on a cell-by-cell basis. Cellular parameters, which are proportional or related to these optical parameters are DNA content, total protein, cytoplasmic granulation (fine structures), nuclear and cytoplasmic diameter among others. These parameters can be determined and displayed as 2 or 3-dimensional frequency distributions. Up to 4 parameters per cell can be stored with the instrument used in the present study.

The use of forward versus right angle light scatter of cells in suspension has been described to differentiate between various cell subpopulations with differing morphology, i. e. size and fine structure (Salzman et al., 1975). As FLC, and especially pulmonary macrophages, demonstrate large variations in cell morphology (Finch et al., 1982) this method can be applied to the various lavaged pools of control and smoke-exposed FLC. For the purpose of differentiating between different cell types, i. e. macrophages and granulocytes, it is preferable to use light scatter parameters because the fluorescence detectors are generally used for the analysis of specific cell properties depending on the assay. There

Einschluß (A)

are 3 light scatter parameters available in the instrument employed in this study:

(1) FWD,

(2) AXL and

(3) RAS.

Each one of these parameters can be analyzed with respect to size and shape of the signal produced by each single cell, i. e. signal area, height and width.

In general, all light scatter signals from cells depend on cell size, absorbance, refractive index and intracellular heterogeneity. This dependence varies with the angle between incident laser beam and detector in an extremely complex manner. There is no generally accepted theory allowing to correlate light scatter to any single specific cell property alone. It is, however, generally agreed that scattered light collected at very narrow angles (0.5 to 2 degrees) relative to the incident beam (so-called forward scatter) mainly reflects cell size with little or no interference by intracellular components. At increasing angles light scatter is more and more affected by intracellular structures, e. g. nuclear size and shape, cytoplasmic inclusions or vacuoles.

In the instrument employed in this study FWD is collected from approx. 2 to 20 degrees (a). At these angles FWD is affected to various degrees by all factors mentioned above. Thus, a clear cut interpretation of this parameter is not possible. Nevertheless, FWD is 1 out of many parameters which can be used to characterize different cell types or changes in a cell population. The use of FWD as an auxiliary parameter is mainly justified by its potential contribution to cell type discrimination.

(a) precise specification not provided by the manufacturer

Einfach (A)

Signals obtained from the AXL detector, which is directly hit by the incident beam of a low power helium-neon laser, are proportional to the amount of light travelling through a cell without being affected in any way. Due to the complex optical properties of cells, however, almost all incident light will be deflected or scattered to some degree. Therefore, cells are essentially dark for this detector. AXL (signal area) can thus be interpreted as a rough measure of the cross sectional area of cells, which, in turn, is a measure of cell size. In this study the signal height of AXL was analyzed because it provided a better separation between macrophages and granulocytes as compared to the signal area. The signal height of AXL can theoretically be expected to be roughly proportional to cell radius raised to some exponent having a value between 1 and 2. A closer definition of the relation between cell size and AXL requires additional methodological studies. Bearing these restrictions in mind, however, a change in AXL can be interpreted as a concomitant change in cell size.

As RAS is strongly enhanced by intracellular heterogeneity it may potentially provide a basis for the determination of vacuolization.

Phagocytosis of particles by pulmonary macrophages has been shown to increase after smoke exposure in previous experiments (a). The method used to date, however, was cumbersome and dependent on cell adhesion which is also reported to vary after smoke exposure (Mann et al., 1971 and Rasp et al., 1978). The determination of FLC phagocytosis in suspension has been described by Kavet and Brain (1977) and this was adapted at INBIFO to fluorescent latex beads as particles, which can easily be quantified by flow cytometry

(a) see REFERENCES: INBIFO studies A 0500/3016 and A 0500/3018

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phagocytosis in suspension has been described by Kavet and Brain (1977) and this was adapted at INBIFO to fluorescent latex beads as particles, which can be easily quantified by flow cytometry (Shellito et al., 1981). This method is not only less cumbersome than short-term adhesion cultures, but also yields information on cell size, number of particles per cell and number of free particles. Furthermore, the phagocytic activity may be corrected for dead cells by the simultaneous determination of viability.

In addition aliquots of FLC were investigated by light microscopy and some biochemical methods.

In the present 21-day inhalation study the influence of sidestream and mainstream cigarette smoke of the standard reference cigarette type 2R1 on selected responses of FLC was investigated.

The objectives of this study were:

(1) the application of flow cytometry in combination with or in place of conventional FLC methods and
(2) the selection of a reproducible lavage method for future studies.

FLC were harvested after a nonrecycling lavage using 10 to 13 cycles. As the composition of FLC populations is strongly influenced by the number of lavage cycles and the constituents of lavage medium, both of these parameters were varied in this study. The lavage media used were:

(1) ~~phosphate buffered saline~~ (PBS),
(2) PBS plus ~~bovine serum albumine~~ (BSA) and
(3) PBS plus calcium and magnesium.

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Biochemical parameters assayed in FLC homogenates were:
(1) protein pattern after separation by electrophoresis,
(2) phospholipid content/macrophage and
(3) specific activity of acid phosphatase.

The FLC parameters assayed by light microscopy were:
(1) proportion of macrophages and lymphocytes,
(2) number of macrophages/rat,
(3) viability of macrophages and
(4) macrophage nucleus and vacuole size.

The FLC parameters assayed by flow cytometry were:
(1) phagocytotic activity of pulmonary macrophages,
(2) DNA content,
(3) viability and nonspecific esterase activity, as well as
(4) differentiation of subpopulations according to light scatter properties (size, structure).

In order to study these parameters 144 rats were randomly allocated to 3 groups, 60 for sham control, 60 for mainstream smoke and 24 for sidestream smoke exposure. The head-only exposure was performed for 420 minutes/day at 0.28 milligrams/liter ~~total particulate matter (TPM)~~ corresponding to a daily dose of 120 minutes x milligram/liter TPM for mainstream smoke and at 0.07 milligrams/liter TPM corresponding to a daily dose of 35 minutes x milligram/liter TPM for sidestream smoke. Rats were exposed 7 days/week for a period of 21 days. This study was divided into 3 subsequent experiments.

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METHOD.

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4 METHOD

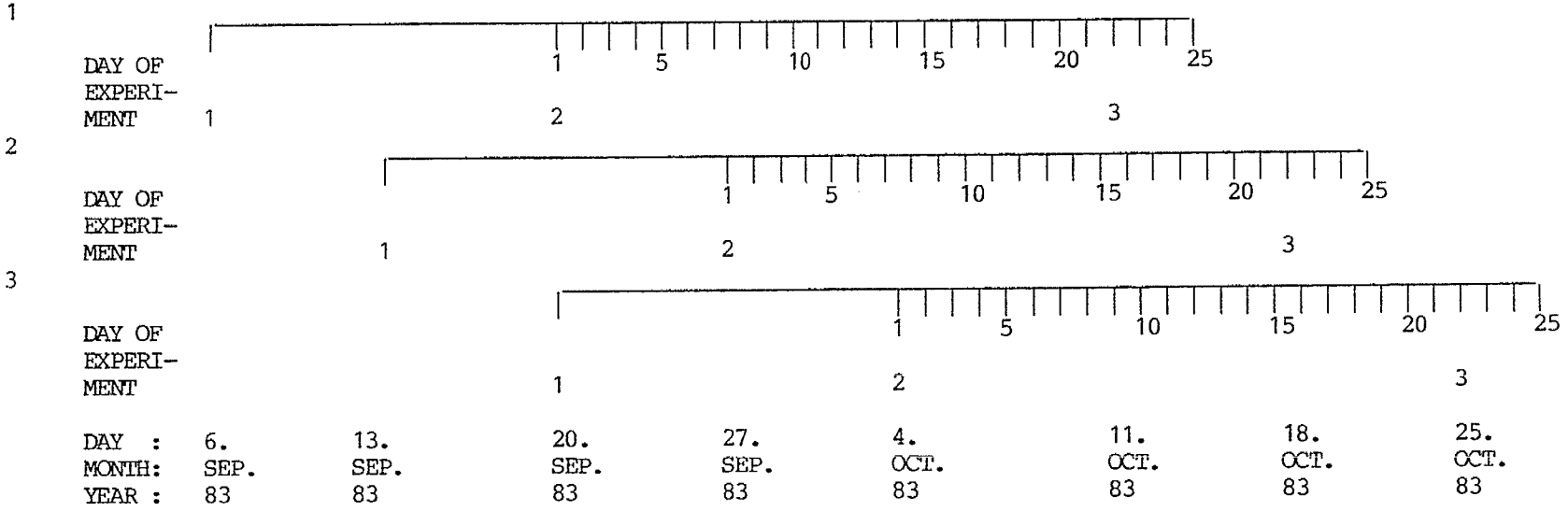
4.1 Chronology (see BC FIGURE A)

4.2 Harvest of Free Lung Cells

Principle:	pulmonary lavage through cannulated trachea
Time:	on day 22
Sample material and quantity:	5 rats per group and day of sacrifice, 3 days of sacrifice (see BC TABLES A and B) pool 1: lavage cycles 1 to 3 with PBS pool 2: lavage cycles 4 to 10 with PBS pool 3: lavage cycles 1 to 3 with PBS plus BSA pool 4: lavage cycles 4 to 10 with PBS plus BSA pool 5: lavage cycles 1 to 3 with PBS plus calcium and magnesium pool 6: lavage cycles 4 to 6 with PBS plus BSA pool 7: lavage cycles 7 to 13 with PBS and BSA pool 8: 3 parts pool 1 and 7 parts pool 2 after adjustment to 1E6 macrophages/ml pool 9: 3 parts pool 3 and 7 parts pool 4 after adjustment to 1E6 macrophages/ml pool 10: 3 parts pool 6 and 7 parts pool 7 after adjustment to 1E6 macrophages/ml
Results expressed in:	-
Equipment:	cannula: stainless steel (V2A), outer diameter: 1 mm, length: 45 mm, sterile, collection vessels: conical glass bottles, 500 ml, silanized, Faust GmbH, D-5000 Köln 90

DETERMINATION OF BIOCHEMICAL AND MORPHOLOGICAL FREE LUNG CELL RESPONSES
groups (a): control : 0.1-GR, 0.2-GR and 0.3-GR
MS-exposed: 1.1-GR, 1.2-GR and 1.3-GR
SS-exposed: 2.1-GR, 2.2-GR and 2.3-GR (b)

1 ARRIVAL OF RATS 2 START OF SMOKE EXPOSURE 3 LUNG LAVAGE, PHAGOCYTOSIS, FLOW CYTOMETRY FIXATION AND/OR WASHING AND FREEZING OF CELLS



BC FIGURE A

CHRONOLOGY

Remarks: smoke exposure: 1-GR : 420 min/d, 0.28 mg/l TPM = 118 min x mg/l 2R1 mainstream
2-GR : 420 min/d, 0.07 mg/l TPM = 29 min x mg/l 2R1 sidestream

(a) 2nd no. of group is code for lavage medium: 1: PBS, 2: PBS + BSA, 3: PBS + Ca/Mg and PBS + BSA
(b) only experiment 3

GROUP	LAVAGE MEDIUM	POOL	CYCLE
0.1-GR	PBS	1	1 to 3
		2	4 to 10
0.2-GR	PBS + BSA	3	1 to 3
		4	4 to 10
0.3-GR	PBS + Ca/Mg	5	1 to 3
	PBS + BSA	6	4 to 6
		7	7 to 13
1.1-GR	PBS	1	1 to 3
		2	4 to 10
1.2-GR	PBS + BSA	3	1 to 3
		4	4 to 10
1.3-GR	PBS + Ca/Mg	5	1 to 3
	PBS + BSA	6	4 to 6
		7	7 to 13
2.1-GR	PBS	1	1 to 3
		2	4 to 10
2.2-GR	PBS + BSA	3	1 to 3
		4	4 to 10
2.3-GR	PBS + Ca/Mg	5	1 to 3
	PBS + BSA	6	4 to 6
		7	7 to 13

BC TABLE A

GROUPS AND LAVAGE PROCEDURE

GROUP	TREATMENT	RAT NO.	DATE OF LAST EXPOSURE	PROCEDURE
0.1.1-GR	sham control	1, 2, 3, 4, 5,	10.Oct.83	lung lavage
0.2.1-GR	"	6, 7, 8, 9, 10,	"	"
0.3.1-GR	"	11, 12, 13, 14, 15,	"	"
0.0.1-GR	"	16, 17, 18, 19, 20,	"	treatment reserve(a)
0.1.2-GR	"	21, 22, 23, 24, 25,	17.Oct.83	lung lavage
0.2.2-GR	"	26, 27, 28, 29, 30,	"	"
0.3.2-GR	"	31, 32, 33, 34, 35,	"	"
0.0.2-GR	"	36, 37, 38, 39, 40,	"	treatment reserve(a)
0.1.3-GR	"	41, 42, 43, 44, 45,	24.Oct.83	lung lavage
0.2.3-GR	"	46, 47, 48, 49, 50,	"	"
0.3.3-GR	"	51, 52, 53, 54, 55,	"	"
0.0.3-GR	"	56, 57, 58, 59, 60	"	treatment reserve (a)
1.1.1-GR	2R1 mainstream	101 102, 103, 104, 105,	10.Oct.83	lung lavage
1.2.1-GR	"	106, 107, 108, 109, 110,	"	"
1.3.1-GR	"	111, 112, 113, 114, 115,	"	"
1.0.1-GR	"	116, 117, 118, 119, 120,	"	treatment reserve(a)
1.1.2-GR	"	121, 122, 123, 124, 125,	17.Oct.83	lung lavage
1.2.2-GR	"	126, 127, 128, 129, 130,	"	"
1.3.2-GR	"	131, 132, 133, 134, 135,	"	"
1.0.2-GR	"	136, 137, 138, 139, 140,	"	treatment reserve (a)
1.1.3-GR	"	141, 142, 143, 144, 145,	24.Oct.83	lung lavage
1.2.3-GR	"	146, 147, 148, 149, 150,	"	"
1.3.3-GR	"	151, 152, 153, 154, 155,	"	"
1.0.3-GR	"	156, 157, 158, 159, 160	"	treatment reserve (a)
2.1.3-GR	2R1 sidestream	201, 202, 203, 204, 205,	24.Oct.83	lung lavage
2.2.3-GR	"	206, 207, 208, 209, 210,	"	"
2.3.3-GR	"	211, 212, 213, 214, 215,	"	"
2.0.3-GR	"	216, 217, 218, 219, 220,	"	treatment reserve (a)
		221, 222, 223, 224	"	treatment reserve (a)
-	hygiene control	601, 602, 603, 617, 618,	-	histopathology
-	"	619, 637, 638, 639	-	"
-	"	604, 605, 606, 607, 608,	-	microbiology
-	"	609, 634, 635, 636	-	"

BC TABLE B

RAT NO., DATE OF LAST EXPOSURE AND PROCEDURE

Remarks: lung lavage approx. 17 h after the end of the last exposure
1st no. of group is code for treatment, 2nd no. for lavage medium and 3rd no. for experiment.

(a) 2 rats from 0-GR and 2 rats from 1-GR will be used for additional histopathological control at the end of each experiment. 3 rats from 2-GR will also be examined at the end of part 3.

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cannula: sterile, no. 9410118,
syringes: 10 ml, sterile, no. 9410010,
2 ml, sterile, no. 9410002,
via Hirtz und Co.,
D-5000 Köln 51

Chemicals:

phosphate-buffered saline (PBS):
Instamed PBS, calcium and magnesium free, no. L182-10, pH 7.2,
PBS Dulbecco with calcium and magnesium, no. L1815,
Seromed GmbH,
D-8000 München 71

bovine serum albumin (BSA),
fraction 5, no. A 4503,
Sigma Chemie GmbH,
D-8028 Taufkirchen

pentobarbital (Nembutal),
CEVA GmbH,
D-2360 Bad Segeberg

lavage media:
(1) PBS
(2) PBS plus calcium and magnesium
(3) PBS plus BSA, final BSA concentration: 3.25 g/l

Procedure

Sacrifice:

rats killed with intraperitoneal injection of a lethal dose of pentobarbital (60 mg/kg body weight)

Lavage:

free lung cells removed from the tracheobronchial and alveolar surface by instillation of lavage medium (approx. 10 ml) under constant pressure of 15 cm water column and free drainage with constant low pressure of 8 cm water column in ice-cooled silanized collection vessels. Instillation and drainage switched by aid of a 3-way stopcock close to the cannulated trachea. Time per lavage cycle: 2 min

Cell storage:	0 degrees centigrade
Scientific version:	SOP BC 174/5
Text version:	17.Aug.82

4.3 Collection of Free Lung Cells

Principle:	centrifugation
Time:	on day 22
Sample material and quantity:	total lavage fluid recovered from 5 lungs, 5 rats per group and day of sacrifice, 3 days of sacrifice (see BC TABLES A and B), pools 1 to 7 adjusted to 1E6 macrophages/ml

pool 1: lavage cycles 1 to 3 with PBS
pool 2: lavage cycles 4 to 10 with PBS
pool 3: lavage cycles 1 to 3 with PBS plus BSA
pool 4: lavage cycles 4 to 10 with PBS plus BSA
pool 5: lavage cycles 1 to 3 with PBS plus calcium and magnesium
pool 6: lavage cycles 4 to 6 with PBS plus BSA
pool 7: lavage cycles 7 to 13 with PBS and BSA
pool 8: 3 parts pool 1 and 7 parts pool 2
pool 9: 3 parts pool 3 and 7 parts pool 4
pool 10: 3 parts pool 6 and 7 parts pool 7

Results expressed in:	-
Equipment:	centrifuge: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 40

pasteur pipettes: silanized,
no. H 55698,
Harshaw,
via Faust GmbH,
D-5000 Köln 90

test tubes: no. 2070, conical graduated, polypropylene, diameter: 30 mm, length: 115 mm,
Becton, Dickinson GmbH,
D-6900 Heidelberg-Wieblingen

centrifuge vessels: conical glass bottles, 500 ml, silanized,
Faust GmbH,
D-5000 Köln 90

Chemicals:

MEM Dulbecco, no. 607789,
Boehringer Mannheim GmbH,
D-6800 Mannheim 31

glutamin, no. 22942,
penicillin, no. 31749,
streptomycin, no. 35500,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

newborn bovine serum, sterile,
no. 29-121-54,
Flow Laboratories GmbH,
D-5309 Meckenheim

cell suspension medium:
MEM Dulbecco, without phenol red,
with 2 mmol glutamin/l,
1E5 U penicillin/l,
7.2E4 U streptomycin/l and
50 ml newborn bovine serum/l

final pH: 7.4

final osmolality: 304 mosmol/kg

Procedure

Centrifugation:

4.9E2 m/s2 (= 500 x g) for 10 min, 0 to 5 degrees centigrade

Removal of supernatant:	from cell pellet with suction pump and pasteur pipettes
Resuspension of cells:	by trituration with silanized pasteur pipettes
Scientific version:	SOP BC 48/2
Text version:	23.Aug.84

4.4 Cell Counting (Hemocytometer Method)

Principle:	microscopic determination of stained cells after lysis of erythrocytes in defined volume of hemocytometer
Time	
Sampling:	on day 22
Determination:	immediately after centrifugation and resuspension of free lung cells
Sample material and quantity:	free lung cell suspension, approx. 5 ul
Results expressed in:	macrophages (1E6/rat)
Equipment:	hemocytometer chamber: Bürker chamber, height of counting chamber: 0.1 mm, no. 9161089, Hirtz und Co., D-5000 Köln 51
	microscope: standard 16, objective: PL 40/0.65, 400-fold magnification, Carl Zeiss, D-7082 Oberkochen
	micro vials: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65

Chemicals:	Türk' solution, no. 9277, E. Merck, D-6100 Darmstadt 1
Procedure:	equal parts of FLC suspension and Türk' solution mixed in a micro vial. After 1 min incubation at RT, sample introduced into the hemocytometer chamber. 3 to 9 square fields counted under a microscope, at least 300 cells.
Scientific version: Text version:	SOP BC 162/2 23.Aug.84

4.5 Determination of Cell Viability (Trypan Blue Method)

Principle:	counting of viable cells which exclude ionic dyes such as trypan blue from the cytoplasm during the 1st 5 min of incubation
Time	
Sampling:	on day 22
Determination:	within 4 h after start of harvest of free lung cells
Sample material and quantity:	free lung cell suspension, 5 ul
Results expressed in:	0/0 viable (= not immediately stained) cells
Equipment:	hemocytometer chamber: Bürker chamber, height of counting chamber: 0.1 mm, no. 9161089, Hirtz und Co., D-5000 Köln 51

microscope: standard 16, objective:
PL 40/0.65, 400-fold magnification,
Carl Zeiss,
D-7082 Oberkochen

micro vials: type "Eppendorf",
polypropylene, no. 3810,
Netheler und Hinz GmbH,
D-2000 Hamburg 65

mixer: Coulter C5,
Coulter Electronics Limited,
D-4150 Krefeld

Chemicals:

trypan blue, no. 1B187,
Chroma Gesellschaft Schmidt und Co.,
D-7000 Stuttgart-Untertürkheim

MEM Dulbecco, no. 607789,
Boehringer Mannheim GmbH,
D-6800 Mannheim 31

glutamin, no. 22942,
penicillin, no. 31749,
streptomycin, no. 35500,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

newborn bovine serum, sterile,
no. 29-121-54,
Flow Laboratories GmbH,
D-5309 Meckenheim

dye solution:
5 g trypan blue/l in cell suspension medium

cell suspension medium:
MEM Dulbecco, without phenol red,
with 2 mmol glutamin/l,
1E5 U penicillin/l,
7.2E4 U streptomycin/l and
50 ml newborn bovine serum/l

final pH: 7.4

final osmolality: 304 mosmol/kg

Procedure:	cell suspension mixed with equal volume of dye solution, cells counted after 5 min of incubation at 0 degrees centigrade number of counted cells: approx. 500
Scientific version:	SOP BC 155/1
Text version:	23.Aug.84

4.6 Determination of Cell Viability and Nonspecific Esterase Activity (Flow Cytometer Method)

Principle:	differentiation and counting of viable and nonviable cells by flow cytometry after incubation with the fluorogenic esterase substrate fluorescein diacetate and the DNA stain propidium iodide, intracellular hydrolysis of fluorescein diacetate yielding green fluorescent fluorescein accumulated by viable cells, propidium iodide entering nonviable cells producing red fluorescence with DNA
Time	
Sampling:	on day 22
Determination:	within 4 h after harvest (a)
Sample material and quantity:	free lung cell suspension in PBS, 250 ul
Results expressed in:	viability: (1) 0/0 viable cells (2) 0/0 intermediate (damaged) cells (3) 0/0 nonviable cells esterase activity: mean green fluorescence, channel number

(a) determinations not always performed within limits due to capacity problems

Equipment:

nytal nylon tissue, 37 um pore size,
Seidengazefabrik AG,
CH-9425 Thal, Switzerland

flow cytometer: Ortho Cytofluorograf,
system 50H with computer model 2150,
argon ion laser coherent, Innova 90-5,
Dr. Molter GmbH,
D-6900 Heidelberg

ultrasonic water bath, Sonorex TK52,
Bandelin KG,
D-1000 Berlin 45

Chemicals:

diacetyl fluorescein, no. F 7378,
propidium iodide, no. P5264,
Sigma Chemie GmbH,
D-8028 Taufkirchen

acetone, no. 14,
E. Merck,
D-6100 Darmstadt 1

phosphate-buffered saline (PBS):
Instamed PBS, calcium and magnesium
free, no. L182-10, pH 7.2,
Seromed GmbH,
D-8000 München 71

diacetyl fluorescein stock solution:
1 mmol/l acetone

substrate solution: 20 ul diacetyl
fluorescein stock solution plus
10 ml PBS, final concentration: 2 um/l

propidium iodide solution:
0.05 g/l PBS

Procedure:

mixing of 250 ul cell suspension with
250 ul propidium iodide solution,
addition of 500 ul substrate solution,
filtration through nylon tissue, incu-
bation at RT, after 10 min sonication
for 2 min, determination of red flu-
orescence (signal area) 15 min after
addition of substrate solution, exita-
tion at 488 nm

Scientific version: SOP BC 207/2
Text version: 23.Aug.84

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4.7 Cell Preparation for Differential Counting

Principle:	application of cells to glass slides by centrifugation
Time	
Sampling:	on day 22
Determination:	within 8 h after start of harvest of free lung cells
Sample material and quantity:	free lung cells, dependent upon number of cells in the lavage medium 100 to 500 ul
Results expressed in:	-
Equipment:	punched filter paper stripes: SCA 005, cytocentrifuge: model SCA 0030, Shandon Labortechnik GmbH, D-6000 Frankfurt 50 slides, no. 9161145, 76 mm x 26 mm, Hirtz und Co., D-5000 Köln 51
Chemicals:	-
Procedure:	cells filled into sample chamber, whose outlet fits into a punched hole of a filter paper strip on top of a glass slide
Centrifugation:	for 10 min with approx. 0.86E2 m/s2 (= 87 x g) at RT 2 slides prepared from each sample
Scientific version: Text version:	SOP BC 165/1 30.Jan.84

4.8 Differential Cell Staining

Principle:	differential staining of cell compartments or components with mixture of acidic, neutral and basic dyes
Time	
Sampling:	on day 22
Determination:	within 1 month after preparation
Sample material and quantity:	free lung cells (cytocentrifuge sediments)
Results expressed in:	-
Equipment:	staining trough: glass, no. 9163281, staining tray, no. 9163500, Hirtz und Co., D-5000 Köln 51
Chemicals:	eosin-methylene blue (May-Grünwald reagent), no. 1424, azur-eosin-methylene blue (Giemsa reagent), no. 9204, E. Merck, D-6100 Darmstadt 1 xylene, no. 8118, Baker Chemikalien, D-6080 Gross-Gerau Corbit-Balsam, I. Hecht, D-2300 Kiel-Hassee eosin-methylene blue: 500 ml/l ethanol azur-eosin-methylene blue: 40 ml/l bidistilled water

Procedure:	according to Pappenheim (panoptic stain) 3 min staining of slides in eosin-methylene blue, 18 min in azur-eosin-methylene blue, intensive rinsing with distilled water and finally air-drying
Scientific version: Text version:	SOP BC 116/1 30.Jan.84

4.9 Differential Cell Counting (Microscopic Method)

Principle:	counting of cells with differentially stained cell compartments or components
Time	
Sampling:	on day 22
Determination:	within 12 month after preparation
Sample material and quantity:	free lung cells (cytocentrifuge sediments)
Results expressed in:	0/0 of the total number of counted cells
Equipment:	microscope: standard 16, objective: PL 40/0.65, 400-fold magnification, Carl Zeiss, D-7082 Oberkochen cell counter: model MF, no. 708238, E. Harig OHG, D-2000 Hamburg 74

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Chemicals:	-
Procedure:	when possible, approx. 300 macrophages, granulocytes and lymphocytes counted 0/0 macrophages (a) = counted macrophages (a) x 100 total number of counted cells
Scientific version: Text version:	SOP BC 10/3 13.Sep.83 [Im Submissionsvorschlag SOP BC 10/4]

4.10 Differential Cell Counting (Flow Cytometric Method)

Principle:	differentiation and counting of cell types according to size and structural heterogeneity by simultaneous measurement of forward light scatter and axial light loss
Time:	variable
Sample material and quantity:	free lung cells in suspension (1) unfixed (2) glutaraldehyde-fixed or ethanol-fixed
Results expressed in:	number of cells per cell type
Equipment:	nytal nylon tissue, 37 um pore size, Seidengazefabrik AG, CH-9425 Thal, Switzerland flow cytometer: Ortho Cytofluorograf, system 50H with computer model 2150, argon ion laser coherent, Innova 90-5, Dr. Molter GmbH, D-6900 Heidelberg

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(a) granulocytes or lymphocytes respectively

	ultrasonic water bath: Sonorex TK52, Bandelin KG, D-1000 Berlin 45
Chemicals:	-
Procedure:	sonication of cell suspension for 5 min, filtration through nylon tissue immediately before flow cytometry, light source: argon ion laser at 488 nm. 2-parameter measurement of forward scatter, signal area, versus axial light loss, signal height, counting of clusters by setting appropriate electronic regions
Scientific version:	SOP BC 210/2
Text version:	23.Aug.84

4.11 Morphometry of Macrophages

Principle:	planimetric determination of macrophage area, vacuoles and nuclei on photographic color slides of microscopical images of stained cytocentrifuge cell sediments
Time	
Sampling:	on day 22
Determination:	within 12 month after preparation
Sample material and quantity:	free lung cells (cytocentrifuge sediments), 2 slides/pool 2 or 3 pools/group

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Results expressed in:

(1) area
macrophages: um2
nuclei: um2
vacuoles: um2 and 0/0 contribution to cell area

(2) number: macrophages, nuclei, vacuoles in classes

CLASS	DIAMETER UPPER LIMIT (um)		
	MACRO-PHAGES	NUCLEI	VACUOLES
0	50.0	30.0	0.10
1	67.5	37.0	0.19
2	91.0	44.0	0.35
3	122.8	51.0	0.65
4	165.7	58.0	1.20
5	223.6	65.0	2.24
6	301.7	72.0	4.16
7	407.1	79.0	7.75
8	549.3	86.0	14.43
9	741.1	93.0	26.86
10	1000.0	100.0	50.00
11	.GT.1000.0	.GT.100.0	.GT.50.00

Equipment:

digitizer: MOP-AM 03,
Kontron Messgeräte,
D-8057 Eching

teletype: ASR 33,
Vollwood,
D-4000 Düsseldorf

Chemicals:

-

Procedure:

tracing of contours of macrophages, nuclei and vacuoles on color slides with digitizer pen

automatic integration of areas

	classification of area according to equal steps on a logarithmic scale
Scientific version:	SOP BC 10/3
Text version:	26.Mar.82

4.12 Silanization of Glass Surfaces

Principle:	covalent binding of silane derivative to hydroxyl groups of glass surface in order to prevent electrostatic cell adherence
Time:	-
Sample material and quantity:	pasteur pipettes or other glass ware
Results expressed in:	-
Equipment:	-
Chemicals:	silicone solution, no. 35130, Serva Feinbiochemica GmbH und Co. KG, D-6900 Heidelberg 1
Procedure:	immersion of glass in silicone solution for 10 s, then in hot air (100 degrees centigrade) for 1 h
Scientific version:	SOP BC 146/3
Text version:	23.Aug.84

4.13 Fixation of Cells in Suspension (Ethanol)

Principle:	denaturation of cellular proteins with ethanol
Time:	on day 22
Sample material and quantity:	free lung cell suspension, variable quantity
Results expressed in:	-
Equipment:	centrifuge: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 40
Chemicals:	ethanol, denatured with ethyl methyl ketone, no. 642, Hofman, D-5000 Köln 21 potassium chloride, no. 4933, potassium dihydrogen phosphate, no. 4873, calcium chloride-2-hydrate, no. 2382, sodium chloride, no. 6404, disodium hydrogen phosphate-2-hydrate, no. 6580, D(+)-glucose-1-hydrate, no. 8342, E. Merck, D-6100 Darmstadt 1 magnesium sulfate, no. M 7506, Sigma Chemie GmbH, D-8028 Taufkirchen Puck' saline G, pH 7.4: 5.4 mmol potassium chloride/l, 1.1 mmol potassium dihydrogen phosphate/l, 1.1 mmol calcium chloride-2-hydrate/l,

	1.4 mmol sodium chloride/l, 1.5 mmol disodium hydrogen phosphate--2-hydrate/l, 5.6 mmol D(+)-glucose-1-hydrate/l, 6.2 mmol magnesium sulfate/l
Procedure:	centrifugation of cell suspension for 10 min at 4.9E2 m/s2 (= 500 x g), 4 degrees centigrade, redispersion of cells in cold Puck' saline G (approx. 1 ml/1E6 cells), slow addition of 3 volumes of cold (minus 20 degrees centigrade) ethanol with vigorous stirring, fixation complete after 30 min on ice storage of fixed cells for max. 2 weeks at approx. 0 to 4 degrees centigrade in ethanol (700 ml/l)
Scientific version: Text version:	SOP BC 208/1 23.Aug.84

4.14 Fixation of Cells in Suspension (Glutaraldehyde)

Principle:	cross-linking of cellular proteins with glutaraldehyde
Time:	on day 22
Sample material and quantity:	cell suspension, variable quantity
Results expressed in:	-
Equipment:	centrifuge: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 40
Chemicals:	glutaraldehyde, no. G6257, Sigma Chemie GmbH, D-8028 Taufkirchen

	phosphate-buffered saline (PBS): Instamed PBS, calcium and magnesium free, no. L182-10, pH 7.2, Seromed GmbH, D-8000 München 71 glutaraldehyde solution: 40 g/l PBS
Procedure:	centrifugation of cell suspension for 10 min at 4.9E2 m/s2 (= 500 x g), 4 degrees centigrade, re-dispersion of cells in cold PBS and addition of the same volume glutaraldehyde solution, fixation complete after incubation of 1 h at 37 degrees centigrade, storage of cells in PBS at 0 to 4 degrees centigrade
Scientific version: Text version:	SOP BC 250/1 23.Aug.84

4.15 Determination of Phagocytic Activity (Flow Cytometric Method)

Principle:	determination of phagocytic activity on the basis of fluorescence/cell caused by phagocytized latex particle measured in flow cytometer.
Time:	incubation: on the day of lung lavage flow cytometry: within 6 weeks
Sample material and quantity:	glutaraldehyde-fixed free lung cells, approx. 5E6
Results expressed in:	(1) number of phagocytizing cells (2) number of nonphagocytizing cells (3) mean number of particles per cell
Equipment:	centrifuges: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 40

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type "Eppendorf", no. 5412,
Netheler und Hinz GmbH,
D-2000 Hamburg 65

nytal nylon tissue, 37 um pore size,
Seidengazefabrik AG,
CH-9425 Thal, Switzerland

flow cytometer: Ortho Cytofluorograf,
system 50H with computer model 2150,
argon ion laser coherent, Innova 90-5,
Dr. Molter GmbH,
D-6900 Heidelberg

microscope: standard 14,
fluorescence microscope with
various filter combinations,
Carl Zeiss,
D-7082 Oberkochen

shaking water bath: WTR-1,
Infors AG,
CH-4051 Basel, Switzerland

Chemicals:

suspension medium:
MEM Dulbecco, no. 607789,
Boehringer Mannheim GmbH,
D-6800 Mannheim 31

glutamin, no. 22942,
penicillin, no. 31749,
streptomycin, no. 35500,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

newborn bovine serum, sterile, no.
29-121-54,
Flow Laboratories GmbH,
D-5309 Meckenheim

glutaraldehyde, no. 4239,
E. Merck,
D-6100 Darmstadt 1

latex particle: fluorescent, mono-
disperse, carboxylated, microspheres,
no. 9847,
Polysciences Inc.,
Warrington, PA 18976, USA

	cell suspension medium: MEM Dulbecco, without phenol red, with 2 mmol glutamin/l, 1E5 U penicillin/l, 7.2E4 U streptomycin/l and 50 ml newborn bovine serum/l glutaraldehyde solution: 40 g/l PBS
Procedure:	opsonizing of latex particle with cell suspension medium incubation of suspended free lung cells with latex particles (10 particles/macrophage) in a final volume of 5 ml at 37 degrees centigrade in a shaking water bath (20 rpm). Reaction stopped after 40 min by cooling on ice fixation with glutaraldehyde solution
Flow cytometry:	sonication of cell suspension for 5 min immediately before determination, selection of cell population using correlated analysis of forward scatter (signal area) and axial light loss (signal height) gated analysis of green fluorescence (signal area, 515 to 555 nm), excitation at 488 nm
Scientific version: Text version:	SOP BC 211/1 23.Aug.84

4.16 Determination of DNA (Flow Cytometric Method)

Principle:	determination of fluorescence by flow cytometry after binding of fluorescent stains to cellular DNA
Time	
Sampling:	variable
Determination:	within 4 weeks

Sample material and quantity:	ethanol-fixed free lung cells in suspension, approx. 1E6 cells/ml
Results expressed in:	(1) fluroescence intensity (channel number) (2) number of cells with specified fluorescence properties
Equipment:	centrifuge: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 40 ultrasonic water bath: Sonorex TK52, Bandelin KG, D-1000 Berlin 45 nytal nylon tissues, 37 um pore size Seidengazefabrik AG, CH-9425 Thal, Switzerland flow cytometer: Ortho Cytofluorograf, system 50H with computer model 2150, argon ion laser coherent, Innova 90-5, Dr. Molter GmbH, D-6900 Heidelberg
Chemicals:	ethidium bromide, no. 21238, mithramycin, no. 29803, Serva Feinbiochemica GmbH und Co. KG, D-6900 Heidelberg 1 tris(hydroxymethyl)aminomethane (Tris), no. T 1503, RNAse, no. R 5503, type I-AS, Sigma Chemie GmbH, D-8028 Taufkirchen sodium chloride, no. 6404, magnesium chloride-6-hydrate, no. 5833, E. Merck, D-6100 Darmstadt 1 ethanol, denatured with ethyl methyl ketone, no. 642, Hofman, D-5000 Köln 21

RNAse solution:
1 g/100 ml PBS

ethidium bromide solution:
0.1 mol Tris/l, pH 7.4,
0.1 mol sodium chloride/l,
0.063 mmol ethidium bromide/l,
final pH: 7.4

mithramycin solution:
7.5 mmol magnesium chloride/l,
125 ml ethanol/l,
0.046 mmol mithramycin/l

Procedure: centrifugation of cells, resuspension in RNAse solution, incubation for 30 min at 37 degrees centigrade, centrifugation of cells, addition of ethidium bromide solution and incubation for 10 min at RT, addition of the same volume mithramycin solution and incubation for 5 min at RT, sonication for 5 min and filtration through nylon tissue prior to flow cytometry, determination of red fluorescence signal height and area, flow rate .LT.200 cells/s

excitation: 457 nm
emission: .GT.500 nm

Scientific version: SOP BC 212/1
Text version: 23.Aug.84

4.17 Determination of Acid Phosphatase (EC 3.1.3.2) Activity

Principle: fluorometric determination of 4-methylumbelliferone at alkaline pH after hydrolysis of 4-methylumbelliferyl phosphate at acid pH

Time

Sampling: on day 22

Determination: within 1 month after sampling

Sample material and quantity:	free lung cell suspension, approx. 5E3 macrophages/assay mixture
Results expressed in:	U/1E6 macrophages or/and U/1E6 FLC (1 U = 1 pmol 4-methylumbelliferone/h)
Equipment:	thermostat: type "Eppendorf", no. 3401, micro vials: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65 spectrofluorometer: model 650-10S, Perkin Elmer GmbH, D-7770 Überlingen rotary mixer: Cenco Deutschland GmbH, D-5667 Haan pH meter: PW 9409, Philips GmbH, D-3500 Kassel shaking water bath: no. 3047, Köttermann KG, D-3165 Hänigsen
Chemicals:	4-methylumbelliferyl phosphate, no. 405663, standards: acid phosphatase from potatoes, specific activity: 2 U/mg, no. 108219, Precinorm E, no. 125091, Boehringer Mannheim GmbH, D-6800 Mannheim 31 sodium acetate, no. 6267, E. Merck, D-6100 Darmstadt 1 glycine, no. 67126, Sigma Chemie GmbH, D-8028 Taufkirchen

quinine sulfate, (standard solution: 1.1 ug/ml sulfuric acid (0.05 mol/l), no. 2-8838,
Carl Roth GmbH,
D-7500 Karlsruhe 21

4-methylumbelliferone, no. 12,872-4,
Ega Chemie,
D-7924 Steinheim/Albuch

bovine serum albumin (BSA), no. 0604,
Baker Chemikalien,
D-6080 Gross-Gerau

Procedure:

according to Robinson, D. and Willcox, P., Biochim. Biophys. Acta 191 : 183-186 (1969)

final concentration of components in assay mixture:

4-methylumbelliferyl phosphate	20 umol/l
sodium acetate, pH 5.0	25 mmol/l
BSA	0.1 to 0.7 g/l

total assay volume: 0.5 ml

incubation time: 40 min

triplicate determination per sample

fluorometric determination:
excitation wavelength: 365 nm
emission wavelength: 448 nm
slit of excitation and emission monochromators: 2 nm

Calculation:

conversion of fluorescence (Frel) into nmol/l of reaction product 4-methylumbelliferone by means of pocket calculator program

standard curve: see BC FIGURE B

Scientific version: SOP BC 141/2
Text version: 23.Aug.84

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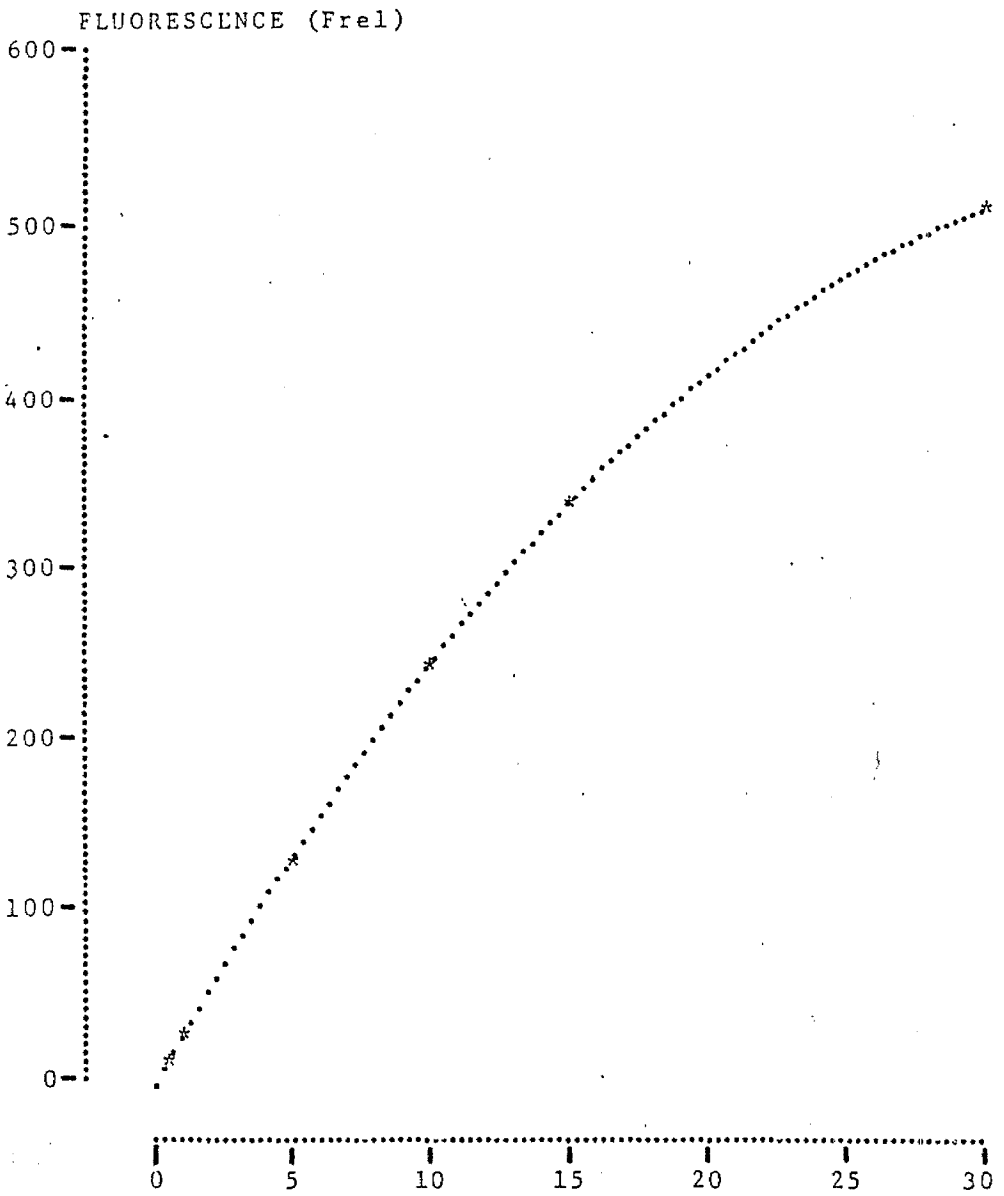

BC FIGURE B

ACP ACTIVITY, STANDARD CURVE FOR DETERMINATION

Remarks: regression curve: Y = AX + BX2 A = 28.339 B = o.372

scientific version: SOP BC 141/2

date of determination: 22.Apr.81

(a) synonym: 4-methylumbelliferyl phosphate (4-MUP)

2.KW83

4.18 Determination of Inorganic Phosphate (Malachite Green Method)

Principle:	photometric determination of phosphomolybdate-malachite green complex at 660 nm
Time	
Sampling:	on day 22
Determination:	within 1 month after sampling
Sample material and quantity:	free lung cell hydrolysate, 500 ul
Results expressed in:	ug
Equipment:	spectrophotometer: model 555, Perkin Elmer GmbH, D-7770 Überlingen
Chemicals:	ammoniumheptamolybdate, no. 1182, hydrochloric acid, no. 317, E. Merck, D-6100 Darmstadt 1 Tween 20, no. 822184, Merck-Schuchardt, D-8011 Hohenbrunn malachite green "G", no. 1B 289, Chroma GmbH, D-7000 Stuttgart stock solution 1: 42 g ammoniumheptamolybdate/l hydrochloric acid (5 mol/l) stock solution 2: 2 g malachite green/l stock solution 3: 15 ml Tween 20/l
Procedure:	according to Itaya, K. and Ui, M., Clin. Chim. Acta 14 : 361-366 (1966)

reaction mixture:
1 aliquot solution 1 mixed with 3 aliquots solution 2, filtered after standing for 30 min

0.5 ml sample mixed with 2.5 ml reaction mixture and 0.1 ml solution 3, photometric determination 5 min after addition of solution 3

photometric determination:
wavelength: 660 nm

standard curve: see BC FIGURE C

Scientific version: SOP BC 179/2
Text version: 23.Aug.84

4.19 Preparation of Fatty Acid Methyl Ester (FAME)

Principle: extraction of phospholipid with chloroform/methanol mixture, hydrolysis and transesterification with methanolic hydrogen chloride and extraction of fatty acid methyl esters with petrol ether

Time

Sampling: on day 22

Preparation: within 10 weeks after sampling

Sample material and quantity: free lung cells, 0.28 to 1.6E6 cells

Results expressed in: -

Equipment: homogenisator: Potter-Elvehjem, B. Braun Melsungen AG, D-3508 Melsungen

centrifuge: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 40

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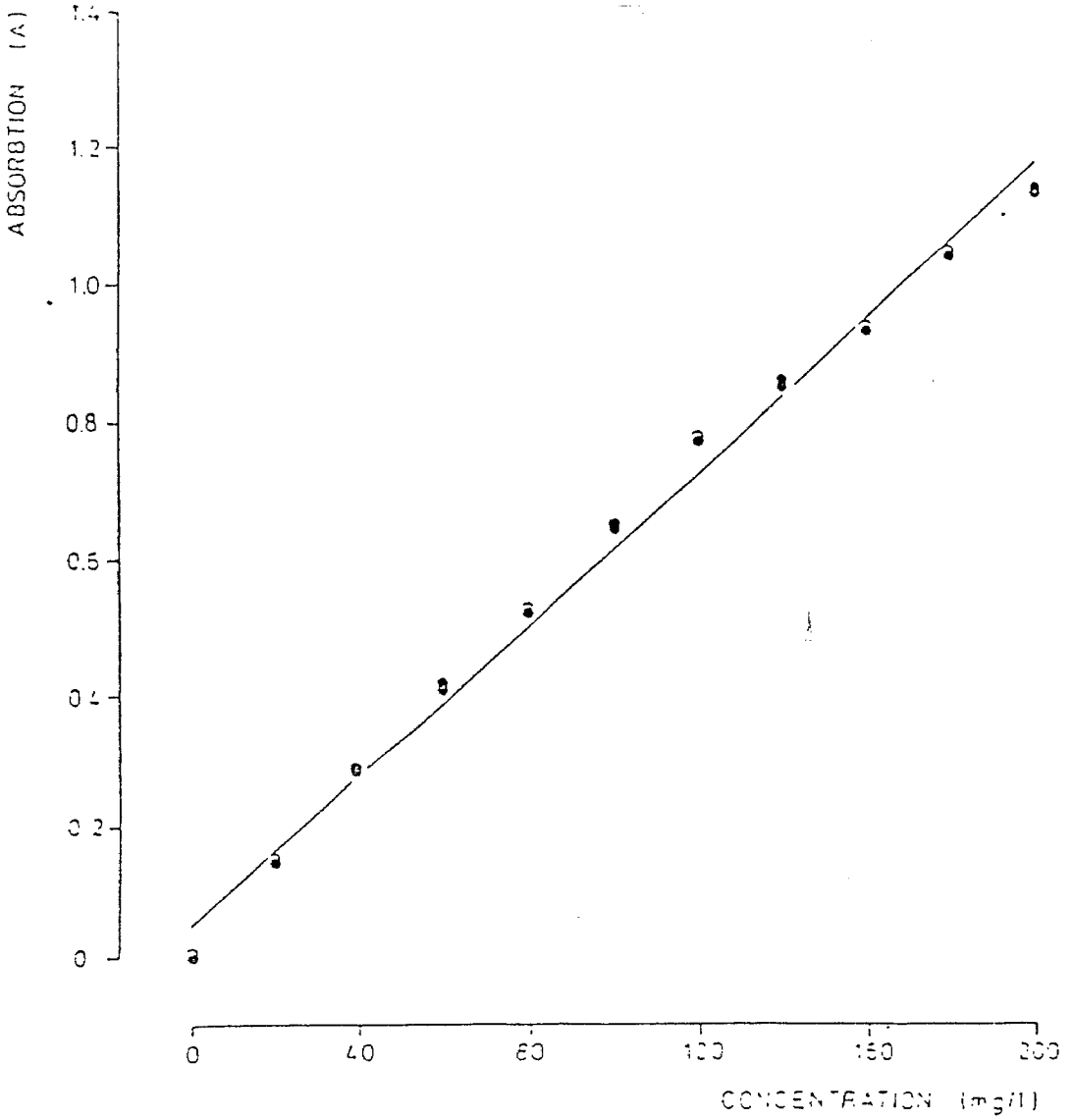

BC FIGURE C

INORGANIC PHOSPHATE, STANDARD CURVE FOR DETERMINATION
(malachite green method)

date of determination: 2.Apr.81

scientific version: SOP BC 179/2

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sample concentrator: Tecum, type SC-3,
Labora,
Karl Heinz Bassler,
D-6395 Weilrod-Winden

Thermostat,
Gebr. Liebisch,
D-4800 Bielefeld 14

vials: 20 ml, 100 mm x 20 mm,
Pyrex with screwcap,
Laborfachhandel

Chemicals:

methanol, no. 6007,
chloroform, no. 2445,
hydrogen chloride, no. 317,
sodium chloride, no. 6404,
hexan, no. 4371,
E. Merck,
D-6100 Darmstadt 1

petroether, 40 to 60 degrees centigrade,
Schnietzler,
D-5000 Köln 41

luvrate methylester, no. 33234,
Chrompack Deutschland GmbH,
D-7840 Mühlheim/Baden

Procedure:

according to Folch, J., Lees, M. and Sloane-Stanley, G.H., J. Biol. Chem. 226 : 497-509 (1957), Schubotz, R., Diagnostic 9 : 200-202 (1976) and Svensson, L., Sisfuntes, L., Nyborg, G. and Blomstrand, R., Lipids 17 : 50-59 (1982)

Homogenization

Extraction: in chloroform/methanol/NaCl mixture

Centrifugation: 9.8E3 m/s2 (= 1000 x g) for 15 min at 4 degrees centigrade

lower phase removed and dried with nitrogen at 25 degrees centigrade

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Hydrolization

Transesterification:	with methanolic hydrogen chloride at 80 degrees centigrade for 1 h extraction of FAME with petrol ether, petrol ether phase dried with nitrogen at 50 degrees centigrade, resuspension of dry pellet with hexan/luvrate methyl ester (internal standard)
Scientific version:	SOP BC 233/1
Text version:	7.May 84

4.20 Determination of Methylated Fatty Acids

Principle:	high resolution gas chromatography of n-hexane extracts of biological material after hydrolysis and derivatization digital integration of peaks, comparison of retention times with pure reference components, determination of response factor and calculation of quantities using internal standard method
Sample material and quantity:	n-hexane extracts, 1 ml
Equipment:	gas chromatograph: Packard 438, detector: FID, injector: SGE II cold on-column, Packard Instrument GmbH, D-6000 Frankfurt/Main capillary column: J. and W., no. 123-5032, ICT Handels GmbH, D-6230 Frankfurt on-column syringe: SGE 5A RN GP CE, Chrompack Deutschland GmbH, D-7840 Mülheim/Baden

integrator/plotter: Shimadzu
Chromatopac GR, B,
Shimadzu GmbH,
D-4000 Düsseldorf

hydrogen,
nitrogen,
synthetic air,
Linde AG,
D-5000 Köln

Procedure

Analysis:	approx. 1 ul injected directly into capillary column at ambient temperature
Gas chromatography	
Column:	30 m x 0.32 mm inner diameter, fused silica deactivated with polysiloane
Stationary phase:	wall coated, DB-5 (a), film thickness: 0.25 nm
Carrier gas and column head pressure:	hydrogen, 0.8 bar (corresponding linear velocity: 55 cm/sec at 55 degrees centigrade)
Make-up gas and flow rate:	nitrogen, 30 ml/min
Oven temperature:	temperature program: 0.5 min at 55 degrees centigrade, rate 10 degrees centigrade/min up to 260 degrees centigrade, 10 min at 260 degrees centigrade
Injector temperature:	ambient, upper part cooled with air
Detector temperature:	325 degrees centigrade
Computation:	gas chromatographic determination of a standard solution of several fatty acid methyl esters, determination of relative response factors using the internal standard method

Scientific version: SOP BC 179/2
Text version: 17.Aug.83

(a) non extractable stationary phase = cross linked and chemically banded silicone containing 5 0/0 phenyl and 95 0/0 methyl groups

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4.21 Preparation of Protein for SDS Polyacrylamide Electrophoresis

Principle:	dissociation of protein subunits with a sulfhydryl compound (beta-mercaptoethanol) and denaturation as well as surface coating with a negatively charged detergent (SDS)
Time	
Sampling:	on day 22
Preparation:	within 7 d after sampling
Sample material and quantity:	FLC homogenate, approx. 1E6 macrophages
Results expressed in:	-
Equipment:	micro vials: type "Eppendorf", polypropylene, no. 3810, thermostat: type "Eppendorf", no. 3401, Netheler und Hinz GmbH, D-2000 Hamburg 65 whirlmix: no. 34526, Cenco Deutschland GmbH, D-5667 Haan analytical balance: model 2001 MP, Sartorius GmbH, D-3400 Göttingen pH meter: PW 9409, Philips GmbH, D-3500 Kassel magnetic stirrer: Ika-Combimag RCO, Janke und Kunkel GmbH und Co. KG, D-7813 Staufen
Chemicals:	disodium hydrogen phosphate-2-hydrate, no. 6580, sodium dihydrogen phosphate-1-hydrate, no. 6346,

beta-mercaptoethanol, no. 805740,
glycerol, no. 4094,
bromophenol blue, no. 8122,
E. Merck,
D-6100 Darmstadt 1

sodium dodecyl sulfate (SDS),
no. 20760,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

iodoacetamide, no. I 6125,
Sigma Chemie GmbH,
D-8028 Taufkirchen

SDS phosphate buffer:
9.75 mmol phosphate buffer/l with
25 ml beta-methaptoethanol/l and
25 g SDS/l

final pH: 7.0

Procedure:

protein incubated for 3 min at 100 degrees centigrade in SDS phosphate buffer, pH 7.0. Thereafter addition of bromphenol blue as tracking dye and glycerol to increase sample density. Further incubation with iodoacetamide at 37 degrees centigrade for 15 min to prevent aggregation of subunits

final concentration of components in incubation mixture:

protein	0.5 g/l
SDS	12.5 g/l
phosphate buffer, pH 7.0	4.88 mmol/l
beta-mercaptoethanol	12.5 ml/l
bromophenol blue	50 mg/l
glycerol	250 ml/l
iodoacetamide	60.4 mmol/l

storage at minus 20 degrees centigrade, stability unlimited

Scientific version: SOP BC 131/3
Text version: 23.Aug.84

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4.22 SDS Polyacrylamide Gel Electrophoresis

Principle:	separation of negatively charged complexes of proteins with a detergent (SDS) acccording to their relative molecular mass in an electrical field across a vertical polyacrylamide gel (separation gel: 125 g/l (= 12.5 0/0) acrylamide, stacking gel: 40 g/l (4 0/0) with defined pore size
Time	
Sampling:	on day 22
Determination:	within 6 months after sampling
Sample material and quantity:	protein SDS complexes, 5 to 40 ul equiv. to 2.5 to 20 ug protein/slot
Results expressed in:	-
Equipment:	maqnetic stirrer: Ika-Combimag RCO, Janke und Kunkel GmbH und Co. KG, D-7813 Staufen pH meter: PW 9409, Philips GmbH, D-3500 Kassel glass cell: Desaga Doppeltrennzelle, thickness: 1.5 mm, width: 220 mm, length: 110 mm, gel volume: 37 ml electrophoresis apparatus: system Havana, Desaga GmbH, D-6900 Heidelberg 1 power supply: no. 2103, LKB Instruments, D-8032 Gräfelfing thermostat: Thermostar RM3, Messgerätewerk Lauda, Dr. R. Wobser KG, D-6970 Lauda-Königshofen

Chemicals:

acrylamide, no. A 8887,
N,N'-methylene-bis-acrylamide (Bis),
no. M 7256,
trizma base (Tris), no. T 1503,
N,N,N',N'tetramethylethylendiamine
(TEMED), no. T 8133,
glycine, no. G 7126,
Sigma Chemie GmbH,
D-8028 Taufkirchen

ammonium persulfate, no. 13375,
sodium dodecyl sulfate (SDS), no.
20760,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

sucrose, no. 7651,
EDTA, no. 8418,
E. Merck,
D-6100 Darmstadt 1

stacking gel composition (40 g/l):

acrylamide	0.561	mol/l
Bis	6.89	mmol/l
sucrose	0.873	mol/l
ammonium persulfate	1.16	mmol/l
TEMED	1.33	ml/l
Tris buffer, pH 6.8	0.125	mol/l
SDS	3.45	mmol/l
EDTA	2.01	mmol/l

separation gel composition (125 g/l):

acrylamide	1.76	mol/l
Bis	21.6	mmol/l
ammonium persulfate	0.583	mmol/l
TEMED	0.665	ml/l
Tris buffer, pH 8.8	0.374	mol/l
SDS	3.46	mmol/l
EDTA	2.04	mmol/l

electrode buffer composition:

Tris	50	mmol/l
glycine	0.383	mol/l
SDS	3.47	mmol/l
EDTA	2	mmol/l

final pH (a): 8.8

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(a) in absence of SDS

Procedure:	glass cells placed in electrode buffer at 9 degrees centigrade current for 2 glass cells: 48 mA for 1 h, afterwards 96 mA tracking dye velocity: approx. 2.5 cm/h
Scientific version: Text version:	SOP BC 142/5 11.Apr.84

4.23 Silver Staining of Proteins (Bio-Rad Method)

Principle:	formation of complex between silver salt and fixed proteins, development of gray to brown color by developer containing paraformaldehyde
Time:	indefinite after fixation of protein in gel
Sample material and quantity:	protein SDS complexes, 5 to 40 ul equiv. to 0.2 to 1 ug protein/slot
Equipment:	magnetic stirrer: Ika-Combimag RCO, Janke und Kunkel GmbH und Co. KG, D-7813 Staufen diffusion destainer, no. 146340, Desaga GmbH, D-6900 Heidelberg 1
Chemicals:	propanol-2, no. 9634, acetic acid, no. 62E, ethanol, no. 983, E. Merck, D-6100 Darmstadt 1

silver stain kit, no. 161-0443,
Bio-Rad Laboratories GmbH,
D-8000 München 50

Procedure:

1st fixation: 1 time for at least 60 min in 250 ml propanol-2/l, 100 ml acetic acid/l

2nd fixation: 2 times for at least 30 min in 100 ml ethanol/l, 50 ml acetic acid/l

oxidation: 1 time for 30 min in 100 ml oxidizer concentrate/l

washing: 3 times for at least 60 min in bidistilled water

staining: 1 time for 30 min in 100 ml silver reagent/l

washing: 1 time for at least 10 min in bidistilled water

1st developing: 1 min in developer solution under constant stirring

2nd developing: 2 times approx. 5 min in developer solution until maximal stain develops

stopping: immediate addition of 50 ml acetic acid/l to last developer solution until no more gas is formed, approx. 5 min

above procedure as suggested by Bio-Rad in accordance with Merril, C.R., Goldman, D., Sedman, S.A., Ebert, M.H., Science 211 : 1437-1438 (1981)

Scientific version: SOP BC 234/1
Text version: 23.Aug.84

4.24 Staining of Proteins (Coomassie Brilliant Blue Method)

Principle:	binding of dye to fixed proteins, removal of excess dye by diffusion
Time:	fixation immediately after electrophoresis to prevent diffusion of protein subsequently stained
Sample material and quantity:	proteins in polyacrylamide gels
Results expressed in:	-
Equipment:	diffusion destainer, no. 146340, Desaga GmbH, D-6900 Heidelberg 1
Chemicals:	propanol-2, no. 9634, acetic acid, no. 62E, Coomassie Brilliant Blue R250, no. 12553, E. Merck, D-6100 Darmstadt 1 methanol, no. 8045, Baker Chemikalien, D-6080 Gross-Gerau
Procedure:	fixation: for at least 15 min in 250 ml propanol-2/l, 100 ml acetic acid/l staining: 30 min in 5 g Coomassie Brilliant Blue/l, 500 ml methanol/l and 100 ml acetic acid/l destaining: 2 to 3 days in 100 ml propanol-2/l, 100 ml acetic acid/l with multiple exchange of destaining solution
Scientific version:	SOP BC 24/3
Text version:	21.Feb.84

4.25 Evaluation of Stained Proteins

Principle:	photometric determination of intensity of protein stain along the electrophoretic separation distance, integration of peak area
Time:	within 2 weeks after staining of polyacrylamide gels
Sample material and quantity:	Coomassie Brilliant Blue R250-stained polyacrylamide gels
Results expressed in:	peak area (arbitrary units)
Equipment:	dual wavelength scanner: model CS-910, Shimadzu Europe, D-4000 Düsseldorf integrator: LDC 301 with printer/plotter, Milton Roy Deutschland GmbH, D-6467 Hasselroth 2
Chemicals:	-
Procedure	
Photometric scanning:	stained gels positioned in the beam of dual wavelength scanner beam focused in the middle of gel slot sample wavelength: 560 nm reference wavelength: 400 nm mode: absorbance, slit size: 1.7 mm x 0.15 mm, scan speed: 20 mm/min
Integration:	fix mode
Scientific version: Text version:	SOP BC 54/2 21.Feb.84